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Development of protein polymorphisms in redwing blackbirds

By ALAN H. BRUSH[1] AND ALAN F. SCOTT[2]
From the Regulatory Biology Section, Biological Sciences Group, University of Connecticut

SUMMARY

Breeding populations of the redwing blackbird, *Agelaius phoeniceus*, were studied to compare protein differences during development. Proteins which represented a minimum of ten loci were studied in egg white, embryonic, nestling and adult tissue. In starch-gel electrophoresis at several conditions of pH only ovoglobulin, ovotransferrin and general esterases were polymorphic. Tissue specificity was observed in other isozymes. These data suggest that developmental sequences of proteins may be considered an adaptative response of the organism.

INTRODUCTION

The existence of protein polymorphisms in avian tissues is now well established (Manwell & Baker, 1970; Karig & Wilson, 1971; Segre, Richmond & Wiley, 1970). However, the function of this variability remains poorly understood (Buettner-Janusch, 1970). A few cases of geographic protein polymorphism have been described in birds (Milne & Robertson, 1965; Brush, 1968), inferring selective advantage in certain environments, and in some cases, preliminary genetic analyses have been attempted (for review, see Ferguson, 1971; Sibley *et al.*, in preparation). Few birds have been studied as thoroughly as the domestic chicken, where extensive polymorphisms have been described in egg white (Baker, 1968) and serum (Baker, Croizier, Stratil & Manwell, 1970).

An aspect of protein variability in avian populations which has been relatively neglected is the sequence in which these multiple molecular systems develop. Although foetal hemoglobins have been described in some detail for several species (Manwell, Baker & Betz, 1966; Shaughnessy, 1970; Borgese & Bertles, 1965; Bush & Townsend, 1971), many questions still remain regarding even this relatively simple system (Wilt, 1967). Other, more comprehensive, developmental studies on serum and other proteins include those on *Gallus* (Manwell & Betz, 1966) and the house sparrow (Bush, 1967; Bush & Siebert, 1968). In both of these cases there were limitations on the numbers and types of proteins and tissues studied and the number of populations sampled.

[1] *Author's address:* Department of Biochemistry, University of California, Berkeley, California 94720, U.S.A.
[2] *Author's address:* Regulatory Biology Section, Biological Sciences Group, University of Connecticut, Storrs, Connecticut 06268, U.S.A.

The present investigation had two objectives. First, to describe proteins at several stages of development in a natural passerine population in order to estimate the degree and possible function of such polymorphisms. The only other species for which descriptions of polymorphisms in various developmental stages is available was the ring-necked pheasant, *Phasianus colchicus* (Baker, Manwell, Labisky & Harper, 1966) and was limited to sampling of an introduced population. Secondly, to determine the presence of polymorphism in a limited number of proteins from different redwing populations, to compare this with material available from other avian species as well as other blackbird species collected incidentally to this material.

MATERIALS AND METHODS

Eggs and embryonic material of *Agelaius phoeniceus* were collected at: the Columbia Wildlife Refuge (Potholes), Othello (Grant County) and Ellensburg (Kittitas County), Washington; and in Mansfield (Tolland County), Connecticut. In the field egg whites and extra embryonic fluids were pipetted directly into capped vials. Small embryos were preserved in phenoxyethanol solution (Karig & Wilson, 1971). Heart, liver and pectoral muscle samples taken from large embryos, nestlings and adults were preserved individually in phenoxyethanol. All samples were cooled in the field and stored at 6 °C in the laboratory.

Starch-gel electrophoresis was carried out in 15% gels at pH 6·0 (Bailey & Wilson, 1968) or 6·8 (Whitt & Booth, 1970) and pH 8·6 (Ashton & Braden, 1961). Electrophoresis was carried out at 4 °C at 2·4–6·0 W. After electrophoresis gels were sliced into two horizontal layers and stained for specific enzymes and other proteins by the methods outlined in Shaw & Prasad (1970).

In order to avoid bias or other problems in phenotype scoring each sample vial was given a unique field number. This was recorded, along with all other pertinent data, in a catalogue. In the laboratory samples were randomized with regard to the order they were run on the electrophoretic gels. Subsequent scoring was done without knowledge of location or nest mates. Thus each sample could be checked against all others as well as itself on subsequent runs.

Embryos were aged according to the standard scheme developed for *Gallus* (Hamilton, 1952). Several problems present themselves in the comparison of a precocial and an altricial species. First, we did not know the absolute chronology of the redwing material. Secondly, embryos small enough to be invisible on cursory inspection in the field were not collected separately. Such samples were marked simply as incubated egg white. As a result of this procedure, we had embryonic material from only the last half of the incubation period or the equivalent of stage 25 of the chick. Finally, because of the many subtle differences in the development of the redwing and the chick, the same stage markers were not of equivalent value. This resulted in some loss of accuracy. As a result we used only

eight Hamburger-Hamilton stages for the blackbird material. Within this framework the staging generally was both consistent and reproducible although it may have lacked some resolution.

Despite the care used in ageing embryos, natural variation in the developmental stage among nest mates appeared frequently. Presumably this was due to developmental variation introduced by different laying times, different rates of development and individual biochemical or genetic variation. Specific cases of variation were observed in the field. For example, nests were found where both eggs and nestlings were present in the same clutch. We also found clutches where some eggs showed obvious signs of incubation and others did not. Finally, there is the variability introduced by geographic variation. Eastern Connecticut populations were approximately two weeks behind those in Washington in their breeding cycle and development.

RESULTS

Egg-white proteins

(A) *Ovalbumin.* No polymorphism was present in the sample of 89 redwing blackbird nests sampled. There was no difference in the mobility of the ovalbumin among the redwing, brewers (*Euphagus cyanocephalus*) or yellowhead blackbirds (*X. xanthocephalus*).

(B) *Ovoglobulins.* Redwing blackbirds were polymorphic at this loci. There were two phenotypes in electrophoresis at pH 8·6. In the populations sampled in 1968 and 1971 the frequencies of the two types were approximately 1:1 (71 nests scored). A similar polymorphism existed in brewers blackbird. However, in this species the frequency of the two types was 5:1 (15 nests).

(C) *Ovotransferrin.* A detailed description of the geographic polymorphism at this locus in the redwing and other blackbird species was published previously (Brush, 1968, 1970). In 62 nests in 1971 the ratio of the M to S allele was approximately 98:2. Local populations monomorphic for ovotransferrin in 1968 were monomorphic in 1971. The percentage of heterozygous individuals in the population was approximately the same in the 1968 and 1971 samples.

Additional collections of brewers and yellowhead blackbird egg white confirmed two observations reported previously. Namely, that brewers, but not the yellowhead, are polymorphic at the ovotransferrin loci. There were only two alleles involved which produce phenotypes that correspond in their mobility to those of the redwing. Secondly, yellowhead blackbirds were monomorphic at the ovotransferrin loci and had an ovotransferrin with an electrophoretic mobility most cathodic of the three blackbird species.

(D) *Lysozyme.* No variation was found in either species and the mobility in all three species was similar.

Embryonic proteins

(A) *Malate dehydrogenase.* Activity of this enzyme was not detected in our material.

Table 1. *Est. 2 phenotypes in embryonic fluid and nestling tissues of redwing blackbirds*

Phenotype	Embryonic fluid	Heart	Liver	Skeletal muscle
Fast (F)	9	2	0	0
Medium (M)	0	1	0	0
Slow (S)	18	1	0	0
F-S	4	0	0	0
M-S	0	1	1	0
F-M	0	3	1	3
F-M-S	0	0	6	0

(B) *Acid phosphatase.* Activity was found in embryonic fluid and embryos from the earliest stages (Hamburger-Hamilton stage 25). It was present as a single broad band which migrated slowly toward the anode at pH 6·8. In advanced embryonic and nestling tissue acid phosphatase was universally present but with a greater activity in liver than in either heart or muscle. No polymorphisms were detected at this locus in a total of 47 samples.

(C) *Lactic dehydrogenase (LDH).* At pH 6·8 all eight samples of embryonic fluid had LDH patterns which consisted of five bands. The primary direction of movement was towards the anode. In the youngest embryos the anodal bands were weak when compared to the cathodal bands of the same individual or anodal bands of more advanced forms. This implied a developmental sequence where the cathodal LDH preceded the development of the anodal type. No polymorphism was detected in this isozyme system.

Embryonic LDH showed marked tissue specificity. Two samples each of liver and heart had LDH patterns which were similar and in which the anodal components stained most strongly on starch gel. Many of the 16 young embryos (stages 25–32) presented only the four most cathodal bands (LDH 2–5). In the 16 advanced embryos (stages 38–40) there was a marked increase in the apparent activity of LDH 1, the anodally migrating band.

In five samples of brewers blackbirds no LDH activity was detected in embryonic fluid. Whole advanced embryos (two animals) had a five-banded pattern similar to the redwing. Liver and heart in developing brewers had strong anodally moving LDH components. Although the sample of ten brewers and five yellowhead blackbirds was considerably smaller than the redwings, the same developmental sequence in LDH isozymes was observed. The five-banded pattern being characteristic of the advanced embryo (stage 34–35 or greater) and nestlings.

(D) *Alcohol dehydrogenase.* At pH 6·0 activity was found only in embryonic liver and heart samples (two samples each). The mobility corresponded exactly with the mobility of the LDH (anodal form). It was not detected in muscle samples.

(E) *Esterase* (Est.). Two quite separate areas of esterase activity were detected. Est. 1 was a relatively slow-moving form. It was present in some samples of embryonic fluid and in the earliest embryos. It appeared as a single band in all tissues, and no polymorphisms were present. Esterase phenotypes were unaffected by gel pH.

Est. 2 appeared as a relatively fast moving complex of bands and was not found in embryos until stage 33–34. Thirty-one samples of embryonic fluid had Est. 2 patterns which appeared to consist of two alleles (Table 1). Inadequate quantities of embryonic tissue were available to determine if tissue specificity existed. The two alleles were expressed as three phenotypes (Table 1). Differences in the Est. 2 phenotype were detected among nest mates. Presumably this reflects the genotypic differences of the parents and may be contrasted to egg-white proteins where nest mates are identical. No Est. 2 allele was associated exclusively with a particular population.

Nestling tissues contained the same Est. 2 alleles as embryos with the appearance of an additional allele, M, in the liver (Table 1). This allele was not present in the most advanced embryos. We presume, therefore, that this allele was unique to hatchlings. The tissue distribution of esterases in nestlings was complex. For example, heart and skeletal muscle commonly had a two-banded pattern, with low apparent tissue specificity. Occasionally an individual would have only one allele present in either muscle or heart tissue. Some individuals had one allele present in one tissue and the alternate allele active in another tissue.

A three-banded liver pattern did not appear until the second day post-hatching. At that point different Est. 2 patterns could be demonstrated in all three tissues of an individual. The esterase patterns of brewers embryos and nestlings were similar in distribution and mobility to those of the redwing.

DISCUSSION

The material in this study is unique because it was drawn from natural populations, in different geographic localities and from several stages in the life-cycle. Unfortunately, some parameters were poorly represented, e.g. embryonic tissues. Nevertheless, there are significant comparative details and information not available for other avian species.

It is important to emphasize the relation of studies of this nature to taxonomic surveys. The conservativeness of proteins, once thought to be a rather broad phenomenon, must be re-established with each protein system and each taxonomic group. Most enzymes show considerable variation in populations (Selander *et al.* 1971), whether for developmental reasons, genetic polymorphism, or physiological variation. This may also be the case among avian plasma proteins (Sibley & Hendrickson, 1970; Feduccia, 1971).

Generalized relationships between egg white and plasma or other adult tissue proteins have been discussed for the ring-necked pheasant (*Phasianus colchicus*) (Baker *et al.* 1966), house sparrow (*Passer domesticus*) (Bush & Siebert, 1968) and domestic chicken (*Gallus gallus*) (Baker *et al.* 1970). In this study we consider not only egg white and selected adult tissues but various embryonic tissues at a variety of developmental stages.

Egg white

Although egg white and serum proteins of chicken show relatively large numbers of polymorphic loci (some of which occur in homologous proteins; Baker *et al.* 1970), the egg white tends to have relatively limited enzymic activity (Baker, 1968). This implies that the proteins involved serve structural, storage or other roles. In populations of the redwing blackbird the pre-albumin, ovalbumin and lysozyme loci were monomorphic. The similarity among the egg-white patterns of redwing, brewers and yellowhead blackbirds confirms the close relationship of these species (Brush, 1970; Sibley, 1970). Polymorphisms were found at the ovoglobulin and ovotransferrin loci. Thus, given the relatively small sample sizes and uneven sampling techniques, these redwing populations appear to be as polymorphic as the quail (*C. coturnix*), where Baker & Manwell (1967) report polymorphisms in 13–14 loci out of 24; 5 of 6 in egg white; and in various strains of *Gallus gallus* where polymorphisms at 5 of 11 loci in egg white were reported (Baker *et al.* 1970).

There is surprisingly little information on frequencies of egg-white protein variants in other species, especially non-Gallinaceous forms, and almost no information on the geographic variation of these avian proteins.

In our studies on blackbirds there were no consistent relationships between allele frequency and specific marsh location within breeding colonies or stage of embryonic development. As in previous samples, heterozygotes at the ovotransferrin loci were not detected in the Connecticut populations. The relative frequency of ovotransferrin heterozygotes detected in the Washington populations did not vary in samples taken three years apart. The significance of this geographical variation remains unclear.

At least ten variants are reported at the G_3 globulin locus for *Gallus*. The polymorphism at this locus, previously unreported in the Icteridae, showed a frequency of the two types (1:1) which did not vary geographically or over several years.

Tissue enzymes

Typical LDH tissue specificity was observed without any polymorphism. The distribution of heart and muscle types agreed with the general picture for birds developed by Wilson, Cahn & Kaplan (1963). The developmental sequence was similar to that reported in *Gallus* (Cahn, Kaplan, Levine & Zwilling, 1962; Lindsay, 1963). The most dramatic changes occurred in the liver, where the amount of MLDH increased in a rather regular manner during development. This

shift, which produces a subunit distribution towards the LDH-5 type, is associated with cells capable of rapid division (Papaconstantinou, 1967). It may reflect rapid liver growth in anticipation of nutritional changes associated with hatching. This interpretation is also consistent with the situation at the Est. 2 locus.

Acid phosphatase and Est. 1 showed no developmental variation nor polymorphism. Malic dehydrogenase activity could not be demonstrated in our material.

In addition to the genetically based variation known or implied in studies on protein polymorphisms, other parameters may influence gel patterns. One such factor is the lack of substrate specificity reported in several animal tissues (Manwell & Baker, 1970). For example, the NBT (nitro blue tetrazolium) stain used for dehydrogenases will react without substrate present (Shaw & Koen, 1968). Further, liver alcohol dehydrogenase in mammals may be artifactual (Beutler, Shaw & Koen, 1967). In the redwing blackbird alcohol dehydrogenase activity coincided precisely with the location of the LDH-1 isozyme. We submit that under these particular conditions of buffer composition and pH the alcohol dehydrogenase activity is an artifact produced by lack of substrate specificity.

Variation among the tissues of individuals and among whole animals at the Est. 2 loci was high and the comparison of patterns complex. If the simplest case was assumed, i.e. minor mobility differences and minor bands are considered irrelevant, then several patterns emerge. The esterases showed some tissue specificity, but individuals varied widely within populations. Within tissues there were varying states of complexity. For example, a unique liver esterase band appeared 2–3 days post-hatching, in contrast to the observation that there are no unique embryonic or adult erythrocyte esterases in *Gallus* (Manwell *et al.* 1966). All four adult redwing tissue samples had esterase patterns generally more complex than those of the embryos or nestlings. Likewise, the plasma of adult and fledgling house-sparrows had more protein bands than did nestlings (Bush, 1967). There were also fewer esterase bands in hatchlings than adult sparrows, again indicative of developmental sequences in individual protein systems. Thus it appears that at least at the loci sampled here intraspecific variation during development was no greater than the genetic polymorphism of adult populations.

The increased esterase banding with age and the shift of LDH near hatching can be attributed to several phenomena. This could be activation (induction) due to dietary shifts, changes in hormonal level or the presence of parasites or disease organisms. The increase in esterases especially may reflect an increased ability to handle a variety of substrates as the environmental challenges presented to the bird becomes more demanding.

These observations strongly suggest that the developmental sequences of the protein systems are adaptative responses in themselves. Presumably studies on avian protein polymorphisms will lead to further insights into evolutionary processes, population biology and phylogeny in much the same way as they have in the mammals (Berry & Southern, 1970).

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